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Title:

Novel imaging method to quantify stratum corneum in dermatopharmacokinetic studies

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Abstract

Purpose: Tape stripping the stratum corneum (SC) is used in topical bioequivalence studies. Formulations are compared using drug concentration profiles as a function of relative SC position; both of these parameters require quantification of SC amount removed per tape. Here, a novel imaging method to quantify SC on tape strips is described. Comparisons are made with established SC quantification methods, specifically weighing and UV pseudo-absorption.

Methods: Six stratum corneum tape strips were measured 15 times by the three methods, which were compared for precision, signal:noise ratio, sample size and speed. Furthermore, 600 tape strips were assayed by each method, and correlations examined.

Results: Weighing exhibited low precision, extremely low signal:noise ratio, and was slow. UV pseudo-absorption had high precision, acceptable signal:noise ratio and was quick. However, only a fraction of the total SC removed is analysed, and inhomogeneity can affect the results. The new imaging method was precise, with high signal:noise ratio, and measured the whole SC sample, unaffected by inhomogeneity. In addition, the approach was rapid and has the potential for rapid automated scanning of multiple tapes and for further image analysis.

Conclusions: The novel imaging method has many advantages over established methods for quantifying SC amount per tape.

Keywords: stratum corneum, imaging, tape stripping, dermatopharmacokinetics

Abbreviations:

SC	Stratum corneum
DPK	Dermatopharmacokinetics
TEWL	Transepidermal water loss

1. Introduction

There is need for an objective method to assess the rate and extent of drug permeation through the skin from topical formulations for bioequivalence purposes. Topical formulations deliver very low levels of drug to the systemic circulation; therefore, drug levels are most appropriately sampled within the skin.

Since the stratum corneum (SC) is the principal barrier to drug absorption, it may be assumed that the (dermatopharmaco)-kinetics (DPK) of drug passage through this layer are related to topical bioavailability [1]. Tape stripping, where layers of SC are removed sequentially with adhesive tapes (after formulation application and removal), has been investigated as a potentially useful method.

When tapes are analysed individually, drug penetration profiles across the SC may be derived. The mass of drug per tape is found after drug extraction and analysis. However, since each tape removes a variable amount of SC, it is logical to measure this quantity on each tape to: (a) express the mass of drug extracted as a concentration, in terms of the 'volume' of SC per tape; (b) measure the depth reached within the SC by each successive tape strip (rather than express the results as a function of tape-strip number; and (c) estimate a volunteer's total SC thickness by tape stripping an untreated adjacent site with concomitant transepidermal water loss measurements [2, 3]; the depth reached within the SC is thus expressed relative to the total SC thickness.

By quantifying the amount of SC per tape, robust, reproducible normalised profiles of drug concentration against relative depth reached within the SC are produced, allowing profiles from different sites and volunteers to be compared [1, 4-11]. Analysis of these profiles, using an appropriate solution of Fick's second law of diffusion, permits parameters describing the partitioning and diffusivity of the drug (reflecting the rate and extent of permeation, respectively) to be estimated and used to compare different formulations.

Traditionally, weighing tapes before and after stripping has usually been used to estimate a mass of SC per tape but, despite refinement of the procedure to improve its 'standardization' [1,3-10,12-14], the process remains time-consuming and laborious. The pseudo-absorption of corneocytes at 430nm has also been used as a relative measure of SC, but inhomogeneous stripping can cause artifacts [15,16]. Other work has investigated quantification of the keratin content of SC per tape strip [17-19], but this method lacks sensitivity to small differences in SC per tape strip, and is a destructive test which is often incompatible with concurrent drug extraction and quantification studies; thus this method is not considered further here.

In this work, a novel imaging method is described, where high-resolution images are taken under highly controlled conditions. The pixels of the image each have an associated greyscale value, which may be measured and analysed. The mean greyscale value across all pixels of the image offers a relative measure of SC per tape. The practical properties of the weighing, UV pseudo-absorption and the imaging methods are compared. Firstly, six tapes were assayed 15 times by each method to measure reproducibility and precision. Secondly, the signal-to-noise ratio of each method was calculated. Thirdly, the proportion of the total tape strip that may be measured was compared. The time typically required to perform each measurement is also discussed.

As the aim of this study was to systematically compare each method to quantify SC per tape, it was decided to take tape strips from a single volunteer to minimize intra-subject variability. In addition, initial tape strips tend to remove more SC per tape; hence, by using a single volunteer, tapes with a wide range of SC per tape were obtained permitting the sensitivity of the quantification methods to be best compared. Furthermore, a series of more than 600 tape strips were then assayed for SC amount by each of the three methods and the correlations between each pair of methods examined.

2. Materials and Methods

2.1 Materials

2cm x 3cm tapes were cut from Scotch Book tape 845 (3M, St. Paul, MN) and stored overnight in covered trays. Tapes were manipulated using a small corner of each tape folded back on itself.

2.2 Subjects

Ethical approval was granted by the Salisbury Local Research Ethics Committee; the Declaration of Helsinki protocols were followed, and written informed consent was obtained from all volunteers. No subject had any history of dermatological disease, or recent topical formulation use.

For the reproducibility assessment, one volunteer (female, 30 years old) provided 6 tape strips from a single site on the volar forearm. For the correlation study, a total of 39 sites, from the volar forearm, were stripped from 10 different volunteers (4 males, 6 females; age range 23-37 years), producing over 600 tape strips.

2.3 Tape-stripping

Two pre-tapes (larger than 2 cm x 3 cm) were applied, with pressure from a weighted roller, to each study site, stripped and discarded. These pre-tapes remove exogenous substances and prepare the skin surface in a systematic way. A plastic template was applied to the skin to delimit a constant area (1.5 cm x 1.5 cm) within the study site. This template had an outline marked on its underside of 2 cm x 3 cm to ensure the tape was always placed in the same position. Six tape strips were taken.

When tape strips were taken for the correlation study, an initial transepidermal water loss (TEWL) measurement was recorded with a closed-chamber evaporimeter (Biox Aquaflux AF102, Biox Systems Ltd., London, UK; measurement range 0–100 g m^2 h^{-1}; resolution ±0.05 g m^2 h^{-1}; probe applied for a minimum 60 s, TEWL obtained as the mean of 10 successive measurements having a CV <1%). A pre-weighed (see below) tape (2 cm x 3 cm) was positioned over the template. Adhesion was ensured with the weighted roller before swift removal. Tape stripping continued with fresh (pre-weighed) tapes, and with intermittent TEWL measurements, until the latter had increased to 4-5 times the original value.

2.4 Techniques to quantify amount of stratum corneum on tape

The amount of SC on each of six tape strips was measured 15 times by each of the three SC quantification methods in turn, as quickly as each method would allow.

2.4.1 Weighing method

Tapes were stored for at least 12 hours before their first weighing. Static electricity was discharged from the tapes prior to weighing (R50 discharging bar and ES50 power supply from Eltex Elektrostatik GmbH, Weil am Rhein, Germany). The tapes were weighed, before and after stripping, using a microbalance with precision of 0.1 µg (Sartorius SE-2F from Sartorius AG, Göttingen, Germany). The mass difference equalled the mass of SC.

Five blank tapes were weighed at the same time as those used for the tape-stripping experiments, both before and after stripping, to correct the calculated mass of SC for any variations in weight due to environmental or other conditions.

2.4.2 UV pseudo-absorption method

After their second weighing, the tapes were mounted on slides (total size 5 cm x 5 cm, internal window 2.3 cm x 3.5 cm) such that a 1.5 cm x 1.5 cm square of SC was centrally aligned with its edges parallel to those of the slide. The mark showing the tape orientation after stripping was always positioned at the same edge of the slide.

The slide was inserted into a specially adapted UV-visible spectrophotometer (Lambda 35, Perkin Elmer, Waltham, MA, USA). The incident light travelled perpendicular to the tape and was delimited by a 1cm x 1cm square screen. The light passed through the tape from the sticky, SC-loaded side. Pseudo-absorption was measured first at 430 nm, and then at 278 nm. A total of four measurements at each wavelength were taken, with the slide rotated through 90° between each one. A blank tape was used to calibrate the instrument before the first tape and after every five tapes. The mean pseudo-absorption was taken as a measure of SC amount.

2.4.3 Imaging method

The slide holding the tape + SC was photographed (14 bit) using a Coolscan V ED slide scanner (Nikon UK Limited, Kingston upon Thames, UK) at a resolution of 4000 pixels per inch (157.5 pixels/mm). Lighting conditions and photographic settings were kept constant. A crop of 2213 x 2203 pixels (approximately 1.4cm x 1.4cm) was centred over the image, using the preview scan, before the full scan was taken.

Cropped images (9822 KB each) were saved and analysed with ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, USA; freeware from http://rsb.info.nih.gov/ij/). A scale of 159.07 pixels/mm was applied to the full image width. During image analysis, the greyscale value of all 4875239 pixels in the image is measured (16 bit) in the range 0-64608, where 0 and 64608 designate black and white, respectively. The *mean greyscale value* across all pixels was calculated and then subtracted from that obtained from blank tapes to yield *mean corrected greyscale values* which increase with image darkness (and hence with the amount of SC on the tape). The mean (corrected) greyscale value is used as a measure of SC amount, such that higher greyscale values correspond to darker images and more SC.

2.5 Statistics

All statistical tests, as detailed with the corresponding results, were performed using Prism® version 4 (GraphPad Software, La Jolla, CA, USA).

3. Results

A typical image of SC on a tape strip, as obtained from the imaging method, is shown in Figure 1. All relevant measurements to obtain the mean corrected greyscale value from the tape are presented in the legend.

Six tapes of standard size (2 cm x 3 cm), with a SC strip area of 1.5 cm x 1.5 cm, were assessed by each method fifteen times, as quickly as each method would allow. These 15 replicate measurements were compared in terms of precision, signal-to-noise ratio, proportion of sample analysed and time "cost".

3.1 Precision

The three methods for quantifying SC were assessed for their inherent precision. Table 1 presents the results obtained. It is important to bear in mind, when comparing the weighing, pseudo-absorption and imaging methods, that quantification of the SC by the weighing approach is not direct; it requires that the tape be weighed twice, before and after stripping, and the SC mass is then deduced from the difference. Thus, the error inherent in the weighing method is doubled. This is different to both the UV pseudo-absorption and imaging methods, which offer direct quantification.

Weighing Method

The relative standard deviation of the mass of the tape is very low, with values typically between 0.009% (tape 06) and 0.050% (tape 04). This represents the error of the balance and is indeed encouraging. However, the mass of the tape *per se* is not of interest.

For each tape, from the 15 repeated measurements, there is always a maximum and minimum mass measured for *blank tape* and *tape with SC* (presented in Table 1). It is thus possible to consider, that when tapes are typically weighed just once for a tape stripping experiment, that extreme weights may be measured by chance. If the minimum mass of the *tape with SC* has the maximum mass of the *blank tape* subtracted from it, the resultant *SC mass* will be low; and *vice versa*. These extremes of calculated *SC mass* are presented in Table 1 for each tape. The differences between the maximum and minimum *SC mass* calculated from these extreme masses of the tapes vary from 0.0326 mg (tape 02) to 0.1404 mg (tape 04). When this is compared to the average mass of SC on a tape, 0.1634 mg, the large error calculating SC mass is now more apparent.

The difference between the maximum and minimum calculated *SC mass* can be related to the average *SC mass* using the 'extreme variability' (Table 1). This value varies between 21.7% (tape 01) and 87.0% (tape 04). Looking at all 6 tapes, the overall 'extreme variability' is ~36% for 15 repeated measures.

It is not possible to calculate a relative standard deviation (RSD) of SC mass. However, the average, rather than extreme, variability can be estimated (labeled [a] in Table 1) and ranges from 3.1% (tape 01) to 10.7% (tape 04). The overall variability, for the six tapes, for these average values is 5.6%.

UV pseudo-absorption

The pseudo-absorption results are presented in a similar fashion in Table 1. It is clear that the precision of the method is very good. Although the absolute values are different between the measurements at 430 nm and 278 nm, there are no significant differences between either the extreme variability or the average measure (RSD) using a paired non-parametric 2-tailed Wilcoxon signed rank test. Consequently, only the results at 430 nm are discussed further.

The extreme variability over the 15 measurements varies between 0.2 and 0.9%, with an average extreme variability of 0.34%. This is ~100 times lower than that of the weighing method (namely

32.5%). The average error, denoted by the RSD, is 0.34% over all 6 tapes, which is ~43 times smaller than that of weighing (5.6%).

Imaging method

The imaging method similarly produces very precise values, with little overall variation in either the VA (0.8%) or the RSD (0.3%) over all replicates of the six tapes (Table 1). These are approximately 39 (= 32.54/0.84) and 21 (= 5.56/0.27) times lower than the corresponding VA and RSD values for the weighing method; and approximately double the variability seen for the pseudo-absorption method.

3.2 Proportion of sample analysed

The weighing method measures all SC removed on the tape.

The pseudo-absorption measurement is restricted to a 1 cm^2 area. The slide holder positions the slides vertically. As such, it is awkward to see if the tape is correctly aligned with the incident beam. To avoid this problem, the SC strip should be at least 1.5 cm x 1.5 cm. The total area measured is 100 mm^2, which is at most 44% of the total SC area sampled (225 mm^2). Previous publications [15,16] used very large tape strips (19 mm x 57 mm = 1083 mm^2) and presumably measured a lower proportion of the total SC. If there is any inhomogeneity, the results from this method will be affected by light scattering [20]; and if inhomogeneous tapes must be discarded (as in [15,16]), the method cannot be generally suitable for assessing the amount of SC.

The imaging method is the most flexible as the crop can be adjusted to fit any size of strip, up to 2.3 cm x 3.5 cm (internal measurements of the slide). In these experiments, a crop of 1.4 cm x 1.4 cm was applied over the 1.5 cm x 1.5 cm strip. This equates to 196 mm^2 measured out of the total of 225 mm^2, i.e., 87.1%.

3.3 Time cost

The weighing method is the most variable in terms of time to complete the procedure, compounded by the fact that each tape needs to be weighed twice. In addition, as the mass of a tape changes with environmental conditions, 3-5 blank correction tapes must be weighed during both weighing sessions. For the 6 tapes, the average time taken to complete the 15 replicate *blank tape* and *tape with SC* weighing was 72 ± 22 min. Both the UV pseudo-absorption and imaging methods are faster, with the 15 replicates performed in approximately 30 minutes for the pseudo-absorption assessment; and 15 minutes for the imaging method (data not shown).

Tape stripping in a DPK study typically requires 20-25 strips per site and experiments usually consist of 2-4 test sites, along with 1 site to assess SC thickness. Obviously, therefore, the weighing method would require a long and unpredictable length of time. The other two methods are quicker, and less likely to vary with environmental conditions. Additionally, the imaging method has the potential for automation using a slide feeder.

3.4 Signal-to-noise ratio

For the correlation study, more than 600 tapes with a 1.5 cm x 1.5 cm square layer of SC were analysed by each of the three methods. The range of values obtained for each method is used to calculate the range of signal:noise ratios.

The 3 cm x 2 cm tapes weigh approximately 50 mg. The range of *SC masses* was 0.025 to 0.25 mg, but more typically in the range 0.05-0.15 mg (Figure 2). Therefore the signal:noise ratio lies somewhere between 1: 200 and1:2000 (Table 2). The fact that the 'noise' of the blank tape is up to 2000 times higher than the signal of the SC undoubtedly contributes to the low precision of this method.

The pseudo-absorption method provides a signal of 0.1 – 0.5 absorbance units for most samples (Figure 2) at 430 nm. Five blank tapes assayed in the same way had a pseudo-absorption of 0.0474 ± 0.0012. This results in a signal to noise ratio of between 1:0.9 to 1:0.1 (Table 2). The signal is thus 1 to 10 times bigger than the noise value, meaning that, for some tapes, the signal may be difficult to distinguish from the noise.

Six blank tapes cropped to an area of 2213 x 2203 pixels, had a mean greyscale value of 64355 ± 29 over a total of 6×4875239 pixels. Approximately 98.5% of all pixels had a greyscale value of 64424 ± 7, which is very close to the absolute minimum of 64608. In order to calculate a signal:noise ratio comparable to the weighing and UV pseudo-absorption methods, all greyscale values, for both the *blank tape* and the *tapes with SC,* were subtracted from the greyscale value of a pure white pixel (64608). Thus, tapes with small amounts of SC are closer in value to the blank tape than tapes with a large amount of SC. The signal:noise ratio of the greyscale method is between 1:0.01 and 1:0.05 (Table 2). This is better than the UV pseudo-absorption method. In addition, the signal is 20-100 times larger than the noise, which means that the signal will not be confused with the background.

3.5 Correlation study

A series of tape strips were collected from intact untreated SC. All mass and greyscale values were collected within a short space of time, and so are analysed together in Figure 2B. For tape set 1 (■), the UV pseudo-absorption scans were also performed at the same time. For tape sets 2 and 3 (● and ×), the UV pseudo-absorption measurements were taken some months after the mass/imaging measurements, and are thus presented separately in Figure 2A and 2C.

Firstly, comparing the weighing method to the UV pseudo-absorption (Figure 2A) a significant positive correlation is seen for all three tape sets, with higher masses of SC associated with higher pseudo-absorption at 430nm. The strength of the non-parametric correlation is similar for all sets with Spearman correlation coefficients, r_s, of 0.53 – 0.57. The pseudo-absorption values are generally reproducible over the four rotations.

It is interesting to note that our correlation coefficient is not as 'good' as that reported in the literature (linear regression (*sic*), r^2 of 0.93 ± 0.05 [16] or 0.92 ± 0.02 [15]). However, this can be explained by the fact that we did not eliminate any tapes from the correlation. We argue that the elimination of tapes on the grounds of "SC inhomogeneity" [15,16] is not useful for the overall aim of using a technique to normalise drug penetration profiles for dermato-pharmacokinetic studies, since all tapes *must* be included for these profiles to provide meaningful pharmacokinetic measurements.

Figure 2B similarly shows a significant positive correlation between the mass of SC on the tapes and the resultant mean greyscale value (Spearman correlation coefficient, r_s = 0.55). There are a large number of data points with a mass of SC between 0.08 and 0.13 mg. This suggests that the gravimetric approach is unable to distinguish between these masses, consistent with our findings on precision and signal:noise. We believe that the resultant poor correlation is mainly because of the lack of sensitivity and large inherent error of the weighing approach rather than being due to the greyscale values. Nonetheless, this lack of correlation is problematic for converting the greyscale values into a workable quantity of SC for normalisation purposes.

Finally, comparing the mean greyscale with the pseudo-absorption, a better correlation is seen, with a Spearman coefficient of 0.75 – 0.98 depending on when the pseudo-absorption measurements were made relative to the imaging measurement. It is not appropriate to perform a linear regression, as neither variable is 'controlled'. However, it can be seen from Figure 2C that the slope of the three groups is similar. This is perhaps not surprising considering the similarities seen when both measurements were correlated with the mass (Figure 2A and 2B). The broad ranges of both the pseudo-absorption and mean greyscale measurements imply that these

methods offer a greater level of sensitivity compared to the gravimetric approach. It is thus easier to distinguish between the quantities of SC on the tapes using these methods. Of course, it is not known whether the quantities of SC are indeed as different as these methods suggest; however, considering the variability inherent to the gravimetric method, and the strength of the correlations ($p<0.0001$), these two methods would certainly be hard to dismiss outright.

Considering the range of values obtained by each method, the mass values also show a lack of sensitivity, with most values in the range of 0.08 – 0.13 mg (factor = 0.62). Both the pseudo-absorption and imaging methods show a wider spread of values, between 0.1 – 0.6 (factor = 6) and 5000 – 25000 (factor = 5) respectively, which will increase their sensitivity for the very slight differences in SC amount on the tapes.

There are differences seen between the correlations depending on when the UV pseudo-absorption measurements were made relative to the other two measurements (Figures 2A and 2B). In Figure 2A, tapes which had their UV pseudo-absorption measured after a delay (sets 2 (●) and 3 (×)) have lower UV pseudo-absorption values for given masses of SC than those measured immediately (tape set 1 (■)). In Figure 2C, tape sets 2 and 3 have a similar slope but different position to tape set 1. For given greyscale values, tape set 2 has lower UV pseudo-absorption values, but set 3 has higher ones. Possible reasons for this are unclear; possibly the tapes have changed with time or the lamp of the spectrophotometer has aged. It is merely important to note that pseudo-absorption measurements should preferably be taken from fresh tapes.

4. Discussion

Comparing the precision of the three methods, it is clear that the weighing approach is less precise than either the UV pseudo-absorption or the imaging methods. If we consider the most extreme 'amounts' of SC that could be derived, the VA for the weighing method is two orders of magnitude higher than that for the other two. Taking instead the error derived from the average 'amount' of SC, the average RSD is fairly similar for the pseudo-absorption and imaging methods, but one order of magnitude greater for the weighing technique.

Although it may seem pedantic to consider these 'worst case' scenarios for the quantification of SC by the weighing approach, the mass can only be measured once during tape stripping experiments due to time constraints. Hence, we do not have the luxury of averaging an outlier value away and these anomalies may occasionally occur. Obviously, when trying to determine very slight differences in mass between different tapes, which is the objective when measuring the quantity of SC during tape stripping, an average error greater than 5% for each tape is far from ideal. In sum, the weighing method to assess SC amount per tape is clearly associated with numerous problems: namely low precision, extremely low signal:noise ratio (up to 1:2000), and a long and variable time needed to obtain measurements. These results call into question the long-term usefulness of this method.

Two alternative methods have therefore been considered concurrently, namely the UV-pseudoabsorption method already discussed in the literature, and a novel imaging method developed in the course of this work. The pseudo-absorption method has high precision, is quick to perform and has an acceptable signal:noise ratio. However, the absorbance of some tape strips is very similar to that of the blank tapes bringing into question the specificity of the method. Furthermore, the area of SC available to be measured is limited to only 1 cm^2. Finally, if the distribution of SC on the tapes is inhomogeneous, the method can be affected by light scattering artifacts [20].

The imaging method seems promising: not only is it very precise, but the signal for the SC layer is high relative to the blank tape. In addition, the method is quick and simple to perform, and is able to measure any sample area up to 2.2 cm x 3.5 cm (i.e., 1 mm less than the internal measurements of the slide holder). Furthermore, permanent records of the SC measurements are obtained, which may be examined further using image analysis software. Future work will aim to apply greyscale values derived from the imaging studies to existing dermatopharmacokinetic models. Specifically, to determine whether the greyscale values can be used to estimate (i) total SC thickness, (ii) cumulative depth reached within the SC, and (iii) the drug concentration within the SC. Dermatopharmacokinetic parameters can thus be calculated and used to compare different topical formulations.

For these experiments, tape strips were taken from healthy, untreated skin on the volar forearm of human volunteers. The SC on the tape strips appeared broadly homogeneous, and a range of values for the amount of SC per tape were determined, allowing the sensitivity of each quantification method to be assessed.

Other commonly used skin samples include: (a) excised (frozen, then defrosted) pig skin; (b) skin treated with topical formulations, and (c) diseased skin. The superior performance of the imaging method should be apparent when studying any of these skin samples. With respect to defrosted pig skin, moisture content can affect the weight of SC measured per tape, and subsequent evaporation can result in the mass of SC decreasing with time, thereby introducing variability. In contrast, the imaging method would not be affected by either moisture content or evaporation. As far as treated skin is concerned, it has been noted that some formulations can affect SC cohesivity, resulting in inhomogeneous layers ("clumps") of SC being removed on a tape strip. In the same way, diseased skin can also produce inhomogeneous tape strips. Once more, while the gravimetric

and UV absorption methods cannot differentiate between homogeneous and inhomogeneous distribution of SC on the tape-strips, the imaging method is able to do so.

5. Conclusions

The low precision, reproducibility and signal:noise ratio of the weighing measurements, coupled with the relatively poor correlations with the pseudo-absorption and imaging approaches, demonstrates conclusively that the weighing method has limited sensitivity for evaluating the amount of SC per tape strip.

From the results presented here, the imaging method appears to be a promising approach to quantify the amount of SC per tape strip. It offers numerous practical benefits and high sensitivity, as well as the potential for automation to scan numerous tapes quickly. In addition, a permanent record of the SC layer is stored, and the images can be examined and further analysed if necessary. In future work, we will consider whether the greyscale values derived from the imaging method can be used as a sensitive relative measure of the amount of SC per tape, as a direct substitute for SC thickness per tape in relevant, existing dermato-pharmacokinetic equations.

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8. Figure legends

Figure 1: A typical image of stratum corneum on a tape strip, quantified with the mean greyscale value measured using the imaging method. Other relevant values involved were: number of pixels = 4875239; mean greyscale value of tape = 46451; mean greyscale of blank tape = 64355; mean corrected greyscale value of tape = 17904; scale (for analysis) = 159.07 pixels/mm; area of strip = 195.1 mm^2.

Figure 2: Correlation of SC measurements by weighing, UV-pseudo-absorption and imaging methods. All correlations are significant ($p<0.0001$) with a 2-tail Gaussian approximation and non-parametric Spearman correlation coefficients (r_s) as indicated. **A.** Weighing versus mean (±SD) UV pseudo-absorption (n = 4 rotations) measured at 430 nm; r_s values are 0.53, 0.53 and 0.57 for datasets 1 (■, n = 202), 2 (•, n = 152) and 3 (▯, n = 249), respectively. **B.** Weighing versus imaging (n = 601); r_s = 0.55. **C.** Imaging versus mean (±SD) UV pseudo-absorption (n = 4 rotations) measured at 430 nm; r_s values are 0.98, 0.85 and 0.75 for datasets 1 (■, n = 202), 2 (•, n = 152) and 3 (▯, n = 249), respectively.

9. Supplemental information

Settings for capturing images using Nikon Scan 4.0.2

Tool Palette Settings

- Crop: *2213 x 2203 pixels*
- Curves: *Off*
- Colour Balance: *Off*
- Un-sharp Mark: *Off*
- LCH Editor: *Off*
- Analogue gain: *Off*
- Scan Image Enhancer: *Off*
- Scanner Extras: *Off*

Scanner

- Film Type: *Positive*
- Scan Colour Space*: Greyscale*
- Bit Depth: *14*
- Transformations: *None*
- Settings: *see under preferences below.*

Preferences (subsequently saved under method file name)

- Gamma: *Off*
- Colour Management: *Use Nikon colour management system*
    - Monitor: *Use factory default*
    - RGB Colour Space: *sRGB*
    - CMYK: *use factory default CMYK profile*
- Single Scan: *All Unselected*
- Batch Scan:
    - *Prompt for information before scan*
    - *Create scan log*
    - *Save to disk*
    - *Stop on errors*
    - *All others unselected*
- File Saving*: Default file format – TIFF*
- Automatic Actions: *All unselected*
- Advanced Colour:
    - *RGB*
    - *White point target: Master 255, Red 255, Green, 255, Blue 255*
    - *Grey point target: Red 128, Green 128, Blue 128*
    - *Black target point: Master 0, Red 0, Green 0, Blue 0*
- Greyscale:
    - *White point target: Greyscale 255*
    - *Blank point target: Greyscale 0*
- Auto-contrast calculations (% excluded):
    - *Black: 0.5*
    - *White: 0.5*
    - *Sample point size: 1x1*
- Preview settings: *all unselected except "cache preview image for single image adapter"*
- Grid settings:
    - *Colour: Black*
    - *Display grid lines every: 10 inches*
    - *Display 4 subdivisions per line*
    - *Unselected: show grid for new windows*

Table 1: Three quantification methods were compared for their variability over 15 repeated measurements, for 6 tapes. RSD: Relative standard deviation.

		Weighing method (mass, mg)			UV pseudo-absorption		Imaging
		Blank tape	**Tape + SC**	**Calculated SC**	**Mean at 430 nm**	**Mean at 278 nm**	**Mean corr. greyscale**
Tape 01	Average **value**	51.8474	52.0286	**0.1812**	**0.4961**	**0.5481**	**21389**
	SD	0.0056	0.0055		0.0005	0.0005	48
	RSD (%)	0.011	0.011	3.07 [a]	0.107	0.092	0.226
	Min value	51.8391	52.0195	0.1616 [b]	0.4955	0.5475	21300
	Max value	51.8579	52.0401	0.2010 [b]	0.4969	0.5489	21455
	Max value - Min value	0.0188	0.0206	0.0394	0.0014	0.0014	155
	Extreme variability (%) [c]			21.7	0.3	0.3	0.7
Tape 02	Average **value**	51.0087	51.1456	**0.1369**	**0.4491**	**0.4939**	**20711**
	SD	0.0053	0.0042		0.0003	0.0003	41
	RSD (%)	0.010	0.008	3.44 [a]	0.058	0.054	0.196
	Min value	50.9996	51.1384	0.1208 [b]	0.4487	0.4935	20638
	Max value	51.0176	51.1528	0.1532 [b]	0.4495	0.4944	20780
	Max value - Min value	0.0180	0.0144	0.0324	0.0008	0.0009	143
	Extreme variability (%) [c]			23.7	0.2	0.2	0.7
Tape 03	Average **value**	48.8321	48.9746	**0.1425**	**0.4584**	**0.5104**	**21466**
	SD	0.0066	0.0065		0.0009	0.0010	59
	RSD (%)	0.013	0.013	4.58 [a]	0.191	0.204	0.277
	Min value	48.8178	48.9664	0.1199 [b]	0.4571	0.5090	21316
	Max value	48.8465	48.9892	0.1714 [b]	0.4597	0.5119	21576
	Max value - Min value	0.0287	0.0228	0.0515	0.0026	0.0029	260
	Extreme variability (%) [c]			36.1	0.6	0.6	1.2
Tape 04	Average **value**	48.7230	48.8843	**0.1613**	**0.4619**	**0.5142**	**21049**
	SD	0.0245	0.0101		0.0003	0.0003	61
	RSD (%)	0.050	0.021	10.74 [a]	0.064	0.064	0.290
	Min value	48.7010	48.8740	0.0715 [b]	0.4614	0.5137	20987
	Max value	48.8025	48.9129	0.2119 [b]	0.4623	0.5146	21191
	Max value - Min value	0.1015	0.0389	0.1404	0.0009	0.0009	205
	Extreme variability (%) [c]			87.0	0.2	0.2	1.0
Tape 05	Average **value**	50.8578	51.0270	**0.1691**	**0.4483**	**0.5150**	**20889**
	SD	0.0091	0.0134		0.0014	0.0020	40
	RSD (%)	0.018	0.026	6.66 [a]	0.321	0.379	0.189
	Min value	50.8459	51.0030	0.1295 [b]	0.4468	0.5130	20809
	Max value	50.8735	51.0564	0.2105 [b]	0.4509	0.5188	20934
	Max value - Min value	0.0276	0.0534	0.0810	0.0041	0.0058	124
	Extreme variability (%) [c]			47.9	0.9	1.1	0.6
Tape 06	Average **value**	50.0620	50.2510	**0.1891**	**0.4809**	**0.5558**	**21831**
	SD	0.0047	0.0137		0.0003	0.0002	90
	RSD (%)	0.009	0.027	4.86 [a]	0.053	0.043	0.412
	Min value	50.0517	50.2316	0.1630 [b]	0.4805	0.5553	21636
	Max value	50.0686	50.2817	0.2300 [b]	0.4814	0.5562	22002
	Max value - Min value	0.0169	0.0501	0.0670	0.0009	0.0009	366
	Extreme variability (%) [c]			35.4	0.2	0.2	1.7
Over all tapes	Ave RSD			5.56	0.13	0.14	0.27
	Average 'extreme variability' (%)			35.99	0.34	0.35	0.84
	Average **value**			0.1634	0.4658	0.5229	21222
	SD **value**			0.021	0.019	0.024	415
	RSD of **value** (%)			12.85	4.08	4.59	1.97

[a] It is not possible to determine a true RSD for the calculated SC mass as this value is found from the measured masses of *blank tape* and *tape + stripped SC*. The values presented are indicative of the variability of the two masses measured, relative to the average SC mass:

$$Weighing\ average\ (\%) = \{0.5 \bullet (SD_{Blank\ Tape} - SD_{Tape + SC})\}/[Average\ SC\ mass]$$

[b] Minimum SC mass per tape is calculated from the *maximum mass* of blank tape, subtracted from the *minimum mass* of tape-with-SC; and *vice versa* for the maximum SC mass.

[c] Extreme variability (%) = 100•{(Maximum SC mass) – (Minimum SC mass)}/[Average SC mass]

Table 2: Calculation of the signal:noise ratio of each method using values for the *blank tape* and the range of *tape with SC* measurements.

	Mean value for *Blank tape* ("noise")	**SC value ("signal")[a]**	**Signal:noise**	**Signal size relative to noise**
Mass (mg)	51.2	0.025	1:2048	
		0.25	1:204.8	
Pseudo-absorption 430nm	0.047	0.053	1:0.887	~1
		0.453	1:0.104	~10
Mean greyscale value[b]	253	24600	1:0.010	~100
		4600	1:0.055	~20

[a] Ranges of values taken from Figure 2, where >600 tapes with SC were measured with each method.

[b] The greyscale value of a pure white pixel is 64608, not 0. All measured values, including mean greyscale of *blank tape*, were thus subtracted from this value before calculating the signal-to-noise ratio.

Figures

Figure 1: A typical image of stratum corneum on a tape strip, quantified with the mean greyscale value measured using the imaging method. Other relevant values involved were: number of pixels = 4875239; mean greyscale value of tape = 46451; mean greyscale of blank tape = 64355; mean corrected greyscale value of tape = 17904; scale (for analysis) = 159.07 pixels/mm; area of strip = 195.1 mm^2.

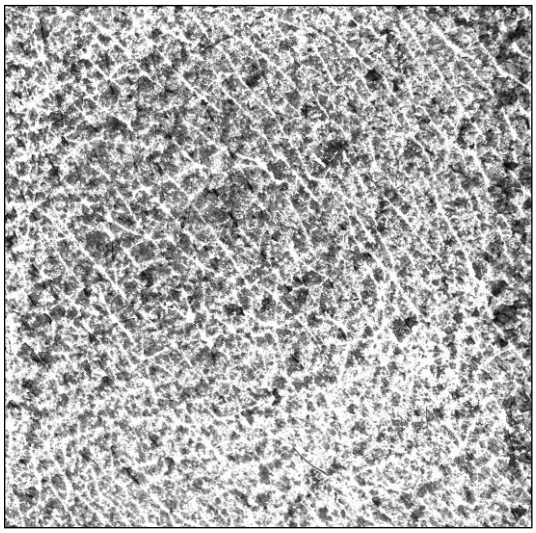
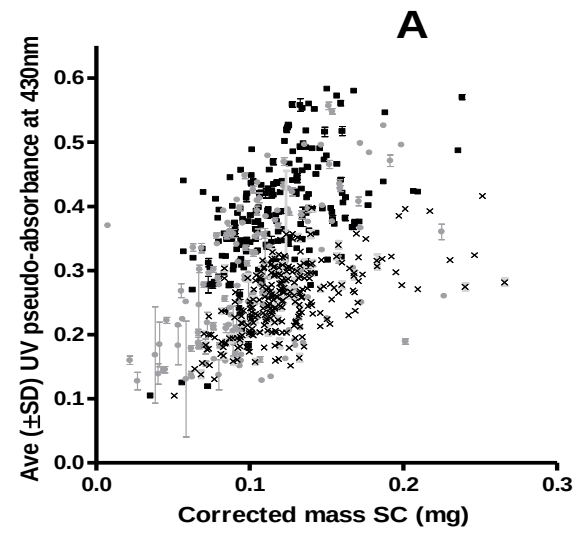
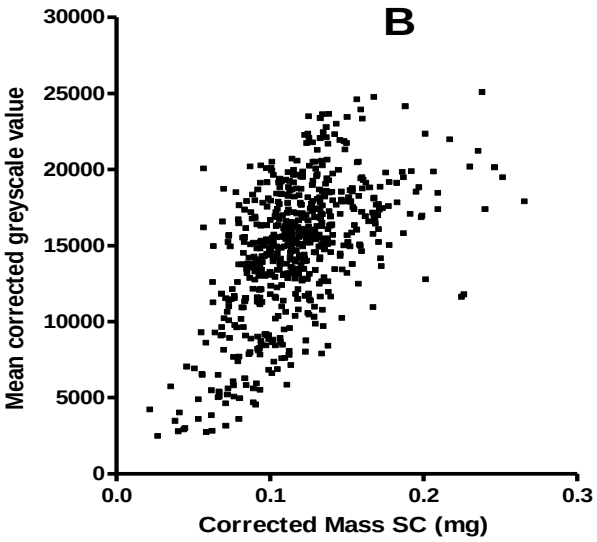
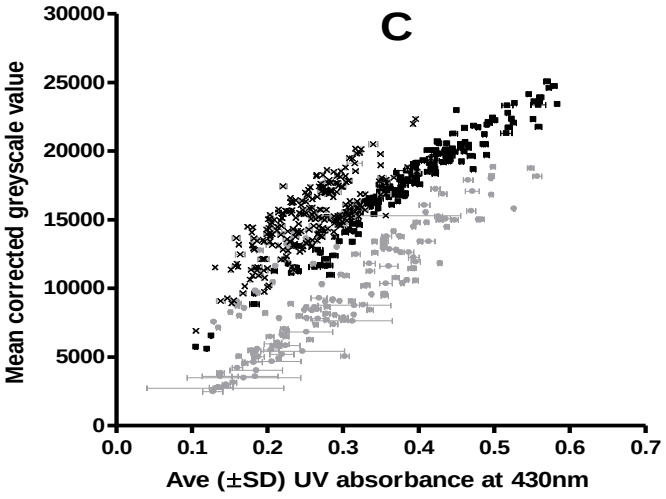


Figure 2: *[See following page]* Correlation of SC measurements by weighing, UV-pseudo-absorption and imaging methods. All correlations are significant ($p<0.0001$) with a 2-tail Gaussian approximation and non-parametric Spearman correlation coefficients (r_s) as indicated. **A.** Weighing versus mean (±SD) UV pseudo-absorption (n = 4 rotations) measured at 430 nm; r_s values are 0.53, 0.53 and 0.57 for datasets 1 (■, n = 202), 2 (•, n = 152) and 3 (▯, n = 249), respectively. **B.** Weighing versus imaging (n = 601); $r_s = 0.55$. **C.** Imaging versus mean (±SD) UV pseudo-absorption (n = 4 rotations) measured at 430 nm; r_s values are 0.98, 0.85 and 0.75 for datasets 1 (■, n = 202), 2 (•, n = 152) and 3 (▯, n = 249), respectively.


A
Ave (±SD) UV pseudo-absorbance at 430nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.0
0.1
0.2
0.3
Corrected mass SC (mg)


B
Mean corrected greyscale value
0
5000
10000
15000
20000
25000
30000
0.0
0.1
0.2
0.3
Corrected Mass SC (mg)


C
Mean corrected greyscale value
0
5000
10000
15000
20000
25000
30000
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Ave (±SD) UV absorbance at 430nm